Large-scale Cultivation of Human Melanocytes Using Collagen-coated Sephadex Beads (Cytodex 3)

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Pure melanocytes were obtained from the epidermis of human foreskin by a modification of a previously described method in which geneticin was added for selective killing of fibroblasts. Purity of the culture was confirmed by light and electron microscopy and by the use of a monoclonal antibody NKI-beteb, which is specific for a vesicular membrane antigen present on melanocytes.

Melanocytes were tested for their affinity to several microcarriers. They attached to cytodex 1 and 3 and dorma cell, but they did not attach to glass and gelatin beads. The best results were obtained with cytodex 3. After an almost immediate and total attachment of melanocytes a fourfold to fivefold increase in cell number was achieved on this microcarrier within 3 weeks. With the results obtained, it seems that the collagen-coated cytodex 3 microcarrier surface supports the growth of melanocytes. Preliminary results obtained with a microcarrier cell culture fermenter clearly indicate that the large-scale cultivation of normal human melanocytes in such an automated system is possible. *J Invest Dermatol 92:18–21, 1989*

Until a few years ago, culturing of melanocytes was considered a difficult task. Recent advances have, however, led to the resolution of several problems associated with melanocyte culture. It is now known that addition of 12–0–tetradecanoyl phorbol 13–acetate (TPA) to a culture medium, containing a mixed cell population from skin, can cause selective plating of melanocytes [1]. Cholera toxin (CT) [1,2], and isobutylmethylxanthine (IBMX) [2,3] can potentiate the mitogenic activity of TPA for melanocytes, and geneticin was shown to prevent the growth of contaminating fibroblasts [4]. Keratinocytes, due to poor plating, disappear during refreshment of medium and subculturing. Besides, several growth factors isolated from certain cells and tissues were able to enhance the viability and proliferation of melanocytes (2,5–7). In spite of these developments, the acquisition of sufficient numbers of melanocytes for certain in vitro studies still remains a problem.

Recent advances in tissue culture technology have revealed that microcarrier cell culture is a technique that has enormous potential for high-yield cultivation of various cell types [8]. Attempts were therefore made to see if melanocytes could be attached and grown on microcarriers. This paper presents the preliminary results on the employment of this technique for the culturing of normal cutaneous melanocytes in an automated fermenter system.

MATERIALS AND METHODS

Chemicals TPA, CT, and IBMX were purchased from Sigma (St. Louis, MO); L-glutamine from Flow Laboratories (Irvine, Scotland); Geneticin (g418-sulphate) from Gibco Europe BV (Breda, The Netherlands); microcarriers, Cytodex 1 and 3 from Pharmacia (Sweden); and dorma cell from Pfeiffer (Dormagen, Germany). Gelatin and glass-bead microcarriers were obtained as gifts from Dr. A.L. van Wezel of the Institute of Public Health (Bilthoven, The Netherlands).

Buffers, Media, Sera and Antisera Ham's F-10, foetal calf serum (FCS) and Dulbecco's modified phosphate-buffered saline (PBS-D) were obtained from Flow Laboratories (Irvine, Scotland). Fluorescein-isothiocyanate (FITC) labeled goat anti-mouse IgG was obtained from Sigma (St. Louis, MO). The monoclonal antibody NKI-beteb was produced in the Netherlands Cancer Institute (Amsterdam, The Netherlands) [9] and kindly provided by Dr. C. Figdor.

Culturing of Melanocytes Melanocytes were cultured by a modification of the method of Eisinger and Marko [1]. Briefly, epidermis from human foreskin was removed with a skin-graft knife (VDL-instrumenten BV, Kaatsheuvel, The Netherlands). It was incubated overnight in 0.25% trypsin at 4°C followed by a short (<5 min) treatment with 0.02% EDTA. The cell suspension obtained was seeded in Falcon Primaria-T-culture flasks ($10–15 \times 10^4$ cells/cm^2) in Ham's F-10 medium (pH 7.2–7.4) containing 5% FCS, 2mM L-glutamine, 16 nM TPA, 0.1 mM IBMX, and 6nM CT (TIC-medium). After 24 h, TIC-medium with unattached cells was withdrawn, and fresh medium supplemented with 100 μg/ml geneticin was added for a period of 3 d. TIC medium was refreshed twice a week. Melanocyte cultures were kept at 37°C in a humidified CO_2-incubator (Hereaus type B5060 EC/CO_2). Subculturing of melanocytes was done after detachment with 0.02% EDTA, 0.005% trypsin in PBS-D in Costar 75 cm^2 culture flasks (seeding density: 10^4 cells/cm^2).

Identification of Melanocytes Melanocytes were identified on the basis of their morphologic characteristics as seen under phase contrast and electron microscope and by immunofluorescent staining of acetone fixed cells with a melanocyte specific monoclonal antibody, NKI-beteb, and FITC-goat anti-mouse IgG.

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Monitoring of Melanocyte Attachment and Growth on Microcarriers In a stationary system attachment of melanocytes to different microcarriers (Cytodex 1 and 3, dorma cell, glass and gelatin beads) was tested as follows: microcarriers (0.4 mg/cm²) were added to melanocytes in a ratio of 1:10 in TIC-medium in flasks. Attachment of melanocytes to the carriers was visualized directly under phase contrast microscopy. Melanocyte growth on the microcarriers was estimated in two different ways: Cells were detached from the carriers of a 1-ml sample with 0.02% EDTA and 0.005% trypsin in PBS-D and counted in a haemocytometer-chamber or by estimation of stained cell nuclei after cell lysis, as described by van Wezel [10].

Migration of undetached melanocytes from a monolayer onto microcarriers was studied by putting the carriers (0.4 mg/cm²) on top of this layer and examining the transport from the culture flask surface to the carriers by daily microscopic monitoring. In a rotating system, growth of melanocytes on cytodex 3 microcarriers was followed while the microcarriers were kept in suspension. Therefore, melanocytes and carriers were combined in a ratio of 10:1 in 25 ml TIC-medium (1mg carriers/ml) in culture flasks. The flasks were gassed with 5% CO_2 and sealed. After the pH of the medium had adjusted to pH 7.2, cells and carriers were transported into 25 ml test tubes, which were then sealed. The test tubes were rotated at 7 rpm on a Cel-Gro Rotator (Lab Line Instruments, Inc., IL) in a 37°C cultivation chamber. Medium was refreshed twice a week after settling down of cells and carriers.

In a cell culture fermenter vessel melanocytes and Cytodex 3 microcarriers (2 mg/ml; 8.0×10^3 carriers/ml) were inoculated in a ratio of 9:1 in 1.2 liter TIC-medium (with 32 nM TPA and 10% FCS). Carriers were kept in suspension with a cell lift impeller at an agitation speed of 22–25 rpm. Temperature (37°C), pH (7.2), and dissolved oxygen (50%) were automatically controlled by the cell culture system (Celligen, New Brunswick Scientific Co. Inc., Edison, NJ). Medium was refreshed batchwise (0.5 l) twice a week after cells and carriers were settled.

Harvesting of Melanocytes from the Microcarriers Melanocytes were detached from the microcarriers by incubation in 0.02% EDTA 0.005% trypsin in PBS-D. The incubation was ended after 5 min in excess of TIC-medium. Cells were separated from the carriers by filtration over a 150-μm screen in a Cellector, tissue sieve (Bellco Glass, Inc., Vineland, NJ). Melanocytes in the filtrate were harvested as cellpellets after centrifugation or used for repeated culture.

RESULTS

Seeding of the foreskin-derived single cell suspension, which consists of a mixed cell population, in TIC-medium resulted in preferential attachment of melanocytes to the culture flask surface.

Fibroblast growth was prevented by the use of Falcon Primaria T-flasks [11] and by selectively killing them by geneticin treatment in an early stage of culture (see *Materials and Methods*). Eventually, pure melanocyte cultures were obtained. Under phase contrast microscope melanocyte cultures showed pigmented and non-pigmented cells with characteristic bipolar and polydendritic morphology (Fig 1*A*). Electron microscopy revealed the presence of intracellular melanosomes in various stages of maturation (Fig 1*B*). IF-staining with the monoclonal antibody NKI-beteb (Fig 1*C*) showed that 100% of the cultured cells were stained.

The degree of attachment of melanocytes to microcarriers after detachment from a culture flask surface is presented in Table I. It could be seen microscopically that melanocytes attach to cytodex 1, cytodex 3, and dorma cell almost immediately after cells and carriers were combined. Few cells were found on the culture flask surface. No such attachment was observed when glass and gelatin beads were used. Spreading of melanocytes on dorma cell and cytodex 3 occurred within 2 to 3 h and was complete within 24 h, whereas spreading on cytodex 1 was incomplete. It was seen that on cytodex 1 part of the attached cells remained nondendritic and spherical and did not look viable.

When cytodex 1 and 3 and dorma cell carriers were seeded onto a monolayer of melanocytes, they attached to this layer. The cells then left the surface of the plastic bottle and migrated onto the microcarriers. This migration process took place much faster and was more complete in the case of cytodex 3 (>90%, day 2 to 6) than for cytodex 1 and dorma cell (<25%, >6 d) (Table I).

In Fig 2*A–C* the whole process of attachment, spreading, and growth on cytodex 3 microcarriers in the stationary system is

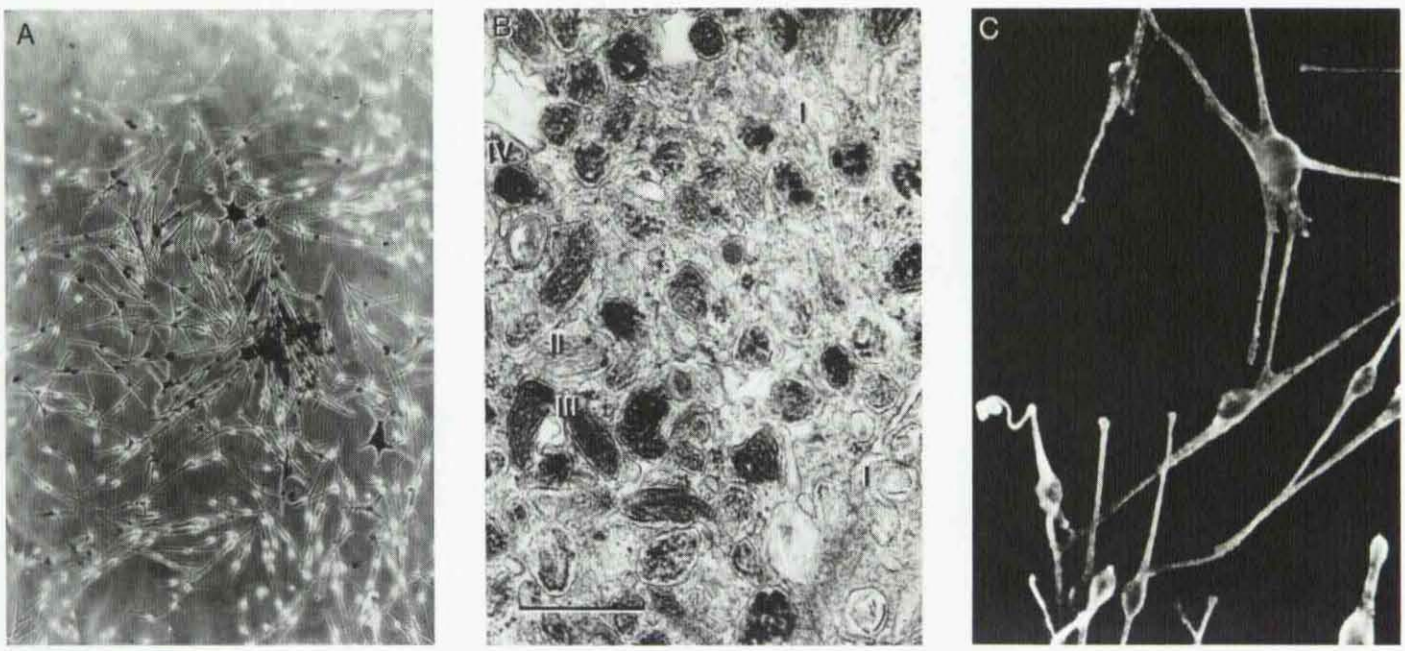

Figure 1. Phase contrast *(A)*, electron- *(B)* and fluorescence *(C)* micrographs of cultured human melanocytes showing partly pigmented cells (third passage) with characteristic bipolar and polydendritic morphology *(A)*, presence of melanosomes in various stages of maturation (indicated by *roman numbers*) in a second passage culture *(B)*, positive immunofluorescent staining of cells (fourth passage) with the MoAb, NKI-beteb *(C)* A: ×100; C: ×200. *Scale bar:* 0.5 μm.

Table I. Attachment of Melanocytes to Different Microcarrier Beads in the Stationary System[a]

Type of carrier	Attachment	Spreading	Migration
Cytodex 1	Direct	Incomplete	<25%, >6 days
Cytodex 3	Direct	Complete	>90%, day 2–6
Dorma cell	Direct	Complete	<25%, >6 days
Glass beads	No	—	No
Gelatin beads	Almost no	No	No

[a] All experiments were done in duplicate.

shown. Although, in the stationary system, after melanocyte attachment to cytodex 1 and 3 and dorma cell, growth was observable on all three types of microcarriers; growth to confluency or near confluency was obtained only in the case of cytodex 3 (between day 14 and 26). Therefore, cytodex 3 was used for testing the growth of melanocytes in suspension in the rotating system. In this system confluency was even more complete and occurred on more carriers at the same time (between day 13 and 18). Also in the 1.2 1 fermenter system good growth was obtained during the first 9 d of culture.

In all cases when confluency or near confluency on microcarriers was reached, detachment of parts of the cell sheet from the microcarriers occurred. In the stationary system this resulted in reattachment to the culture flask surface. In the rotating systems these unattached cells were harvested at medium refreshment and could be used for replating in culture flasks.

For the three different systems, the stationary flasks, the rotating test tubes, and the fermenter system, the maximal average cell to carrier ratios that could be achieved were 30, 50, and 23 cells per carrier, respectively. These maximal average cell-to-carrier ratios were achieved after 26 d in the stationary system, 18 d in the rotating test tubes, and 9 d in the fermenter system. From the known carrier concentrations (mg/ml; see *Materials and Methods*) and the approximate carrier area of 4.6 cm^2/mg (as given for cytodex 3 by Pharmacia, Sweden), the maximal melanocyte densities were calculated for the three systems. These results are presented in Table II and compared with the normal culture situation in culture flasks without microcarriers. Table II shows that in the stationary system a cell-to-carrier ratio of 30 to 1 and a cell density on the carrier surface of 2.6×10^4 cells/cm^2 were obtained. This density is comparable to that obtained on the culture flask surface (3.0×10^4 cells/cm^2).

The 0.4 mg cytodex 3 added per cm^2 culture flask surface resulted in 84% surface enlargement (1.84 cm^2 carrier area/cm^2) and 60% higher cell densities per ml medium in the culture flasks (28.8×10^4 compared to 18.0×10^4 cells/ml). Because starting densities were known in all systems (see *Materials and Methods*) and the maximal cell densities were reached after different periods of time, average increases per day were calculated to compare cell yield per ml medium per day in the different systems. In Table II it can be seen that in the stationary system with microcarriers the daily increase in melanocyte density was higher than without the microcarriers (7.4 and 5.7×10^3 melanocytes/day/ml, respectively). In the rotating test tubes, where the optimal density of 50 cells/carrier was obtained, the average increase was even better (8.9×10^3 melanocytes/day/ml). The completely confluent microcarriers contained 4.3×10^4 melanocytes/cm^2 carrier surface, which is 43% higher than on the culture flask surface.

Although in the fermenter system a suboptimal density of 23 cells/carrier was reached, the higher carrier concentration in the fermenter system (2 mg/ml compared to 1 mg/ml in the test tubes) and the fast growth during the first 9 d of culture resulted in the highest average daily increase in cell density (12.4×10^3 melanocytes/day/ml), a total amount of 220.8×10^6 cells in the 1.2 l fermenter and a total increase in cell number of 134.4×10^6 melanocytes.

DISCUSSION

We have shown in these experiments that pure melanocyte cultures could be obtained from human foreskins under the described conditions. The purity could be ascertained on the basis of morphologic characteristics, the presence of melanosomes in all the cells viewed under electron microscope, and the positive reaction of 100% of the cells with the monoclonal antibody NKI-beteb, which was directed against a vesicular membrane antigen specific for melanocytic cells [9]. Results obtained with several types of microcarriers showed that melanocytes had the highest affinity for collagen I-coated cytodex 3. This was in accordance with the findings of Gilchrest et al [12] who reported rapid attachment and spreading of melanocytes on collagen types I and III coated surfaces. Cytodex 3 proved superior to other carriers with respect to viability and growth of melanocytes. The dextran surfaces of the cytodex 1 and the dorma cell carriers positively charged with mono and dimeric N,N-diethylaminoethyl (DEAE) groups, respectively, seem to be less favorable for melanocyte culture.

The growth of melanocytes on cytodex 3 had several advantages. Seeding of the carriers in culture flasks in a concentration of 0.4 mg/cm^2 resulted in surface enlargement and a 60% increase in cell density per culture flask (see Table II). Because part of the cells detached from the microcarriers at confluency and reattached to the

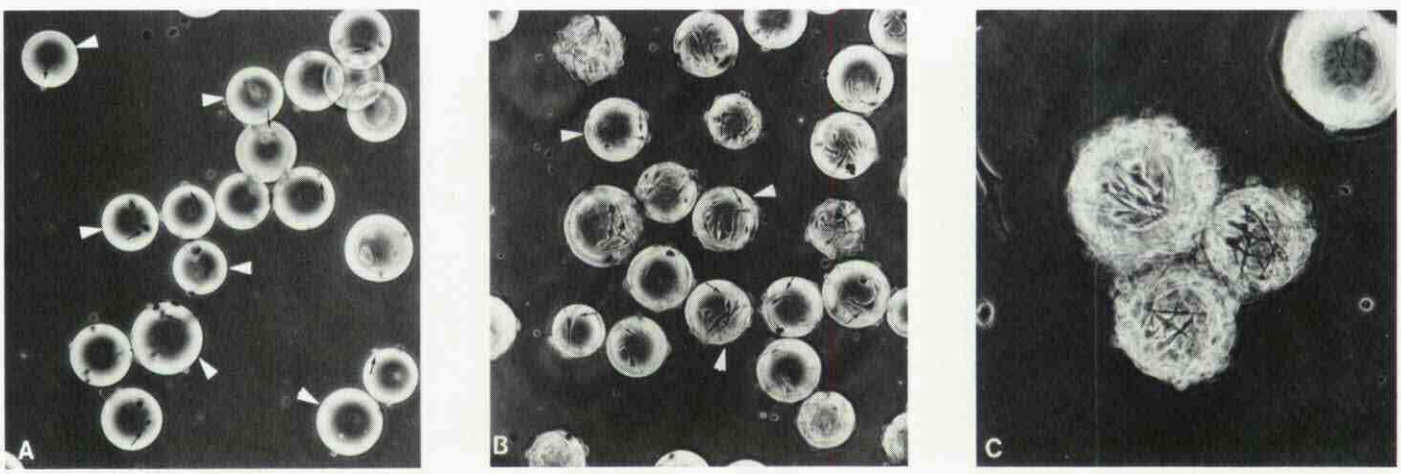

Figure 2. Melanocytes (fifth passage) *(arrows)* attach to cytodex 3-microcarriers *(arrowheads)* within a few minutes after they were combined *(A)*. Spreading of the cells on the carriers is complete within 24 h *(B)*. Growth of melanocytes on the carriers resulted in high cell densities after 14 d of culture under static conditions in flasks *(C)*. Phase contrast micrographs: *A* and *B*: ×100 and *C*: ×200.

Table II. Melanocyte Growth in Four Different Systems

System	Time (days)	Maximal Melanocyte Density per carrier	Maximal Melanocyte Density per cm^2 ($\times 10^{-4}$)	Maximal Melanocyte Density per ml ($\times 10^{-4}$)	Average Increase in Melanocyte Density per Day per ml ($\times 10^{-3}$)	Total Achievable Amount of Melanocytes per Culture Unit ($\times 10^{-6}$)
Stationary						
Flasks[a]	21	—	3.0[b]	18.0	5.7	2.2/flask
Flasks + carriers[a,c,d]	26	30	2.6[e]	28.8	7.4	3.6/flask
Rotating						
Test tubes[d,f]	18	50	4.3[e]	20.0	8.9	5.0/tube (25 ml)
Fermenter[d,g]	9	23	2.0[e]	18.4	12.4	220.8/vessel (1.2 l)

[a] 12.5 ml culture medium was used for one 75 cm^2 Costar-culture flask.
[b] per cm^2 culture flask surface.
[c] 0.4 mg cytodex 3 microcarriers were seeded per cm^2 culture flask surface (= 2.4 mg.ml medium^{-1}).
[d] approx. no. of cytodex 3 microcarriers per mg = 4.0×10^3.
[e] per cm^2 carrier area (cytodex 3 : 4.6 cm^2.mg^{-1}).
[f] 1.0 mg cytodex 3 microcarriers.ml medium^{-1}.
[g] 2.0 mg cytodex 3 microcarriers.ml medium $^{-1}$.

culture flask surface the total cell density reached was even higher than 28.8×10^4 per ml (Table II). In this stationary system the carrier concentrations could be increased to cover the complete culture flask surface. Higher yields per cm^2 may be obtained in this way.

In the rotating test tubes almost complete confluency was reached on all the carriers resulting in a melanocyte density approximately 43% higher than on the culture flask surface. Starting with a lower cell density than in the culture flasks a higher density was reached (20.0 to 18.0×10^4 melanocytes/ml) in a shorter period, which implies that growth on the collagen I-coated carrier surface is better than on the culture flask surface.

The results obtained in the fermenter system proved that enormous quantities of melanocytes can be obtained in a very short time. The maximal amount achieved after 9 d of 220.8 million cells is comparable to the amount one can obtain in 100 culture flasks (see Table II). Maintaining such large quantities in culture flasks would be difficult because of the amount of labor involved, the high risk of contamination, and because a lot of space would be needed for storage of the flasks. When all these cells are cultured in one 1.2 l fermenter, the risk of contamination from medium refreshment and cell harvesting is drastically reduced. This also reduces the amount of labor involved.

Until now, no higher cell densities per carrier than 23 were obtained in the fermenter. This may be due to an uneven distribution of cells over the carriers during the inoculation procedure resulting in confluency on the microcarriers at different times. Another reason could be a shortage of nutrients and/or accumulation of toxic metabolic products in the medium. Higher agitation speed and carrier concentrations during inoculation may result in the more-even distribution of melanocytes over the carriers. Extra additions of amino acids, vitamins, pyruvic acid, glucose, and attachment factors or medium refreshment by perfusion may overcome the problem of changes in the medium.

With these improvements a cell-to-carrier ratio may be obtained near the optimal values reached in the test tubes. Together with higher carrier concentrations this could lead to at least a twofold increase in cell number in the 1.2 l fermenter. Scaling up to a 2.5-l vessel may provide us with cell numbers in the order of 10^9. In our case these large quantities of melanocytes will be used for enzymologic investigations of melanin metabolism, but they can be used for many other biochemical and immunologic studies on this differentiated cell type which are so far not reported in the literature.

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